THE ORGANISM OF RABIES AND EXPERIMENTS IN ITS ARTIFICIAL CULTIVATION.*

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The following experiments were carried on with a hope of securing some evidence which might aid in establishing the true nature of the Negri bodies. It has been frequently observed that, in the earlier stages of rabies, the Negri bodies either may not be demonstrable at all, or they may be few and minute, while in animals which have died of the disease the bodies are larger and more numerous. To trace this development through successive stages if possible was the purpose of the first series of experiments.

Dogs were inoculated with street rabies by the direct injection of emulsion of rabid brain into a nerve trunk. Various nerves were employed, but most satisfactory results were obtained by introducing a long needle into the orbital cavity below the eyeball and making the injection at the point where the optic nerve emerges through the optic foramen, the operation being made under ether anesthesia. The animal was then observed closely and at the first sign of unusual restlessness or excitability, usually after 12 to 18 days, it was killed and the brain taken out under the most careful aseptic precautions. Sections about 3 millimeters in thickness were taken through Ammon's horn and adjacent tissue, and incubated in sterile dog serum in tubes with a layer of olive oil above the serum. The olive oil served the double purpose of preventing evaporation and producing partial anaerobic conditions. Before placing the brain tissue in the tubes a small bit of gray matter was cut from each section and a smear made as a control for future comparison. At intervals of 24 hours tubes were opened and specimens made and compared with their respective controls.

The chief difficulty lay in determining the stage at which the dog should be killed and the incubation *in vitro* begun. When this stage was chosen opportunely either no Negri bodies would be seen

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in the freshly stained preparation, or the forms would be very minute, ranging from 1 to 3 microns in diameter. Marked increase in both number and size was evident in 24 hours in most cases and the development continued until the tissues became so degenerated from incubation that satisfactory staining was difficult. After 48 or 72 hours the larger bodies attained a diameter of from 6 to 9 microns while definite increase in numbers was evident, and the smaller forms were present in abundance. The bodies showed the characteristic dark granules and staining properties, but greater irregularity of form was evident than the Negri bodies show under ordinary conditions. Controls of normal brain tissue incubated under the same conditions showed no forms which would be mistaken for Negri bodies by an experienced observer. Equally satisfactory results were obtained when the sections were incubated in Ringer's solution instead of serum.

These results lend additional support to the view that the Negri bodies are forms in the life cycle of a protozoan organism as against the view that they are the results of the reaction of the living nerve cells to the disease. The increase in the size and the number of the bodies perhaps might result from a progressive chemical change in the brain substance, but the notion that the bodies are the results of the reaction of the living cell toward the unknown parasite or its toxin, is hardly consistent with the results of this experiment.

Cultivation of the supposed organism was attempted as follows: the medium used was fresh brain matter taken from a normal dog under the most careful aseptic precautions. The brain substance was proved sterile by incubation and subcultures. The following media were tried:

Brain material under sterile dog serum and oil;
" " " " cerebrospinal fluid and oil;
" " " Ringer's solution and oil;
4 parts plain agar+1 part brain substance thoroughly mixed;
1 part brain+9 parts salt solution, emulsified by shaking with glass beads, and filtered through a coarse Berkefeld filter.

This filtrate under a layer of sterile oil was used as a culture medium.

Inoculations were made with a drop of emulsified rabid brain substance, and the medium incubated at 38° C. for from 8 to 15

days, both aerobically and under strict anaerobic conditions produced by a combination of vacuum, displacement by hydrogen, and absorption of oxygen by KOH+pyrogallic acid. Smears were then made and stained with rosanilin violet+methylene blue as described by Williams and Lowden.[1]

Guinea-pigs were inoculated with the medium after incubation as a test for virulence. As a control to determine how far the original infective material might be responsible for virulence of the subcultures, a series of tubes containing salt solution under oil were inoculated and subcultures made, incubated, and tested for virulence in the same manner as the culture tubes. The tubes of brain agar and brain emulsion filtrate gave no encouraging results either anaerobically or aerobically. The tubes of brain substance in dog serum, cerebrospinal fluid, and Ringer's solution, however, gave some encouragement, and as the results in these three suspension media were practically the same, Ringer's solution was adopted as a medium and the use of dog serum and cerebrospinal fluid was discontinued. Strict anaerobic conditions were also discontinued as unnecessary and only the partial exclusion of oxygen by olive oil or paraffin oil was practiced.

In microscopic examination of the cultures many bodies were found which resembled Negri bodies, but due to the degenerated condition of the brain substance following incubation it was impossible to say that these might not be degenerated cell nuclei or other artifacts. This being the case reliance was placed mainly on the results of animal inoculation. This was performed by introducing a drop of emulsified culture material subdurally through a small opening drilled through the skull of a guinea-pig. The inoculation was made under ether anesthesia and surgical asepsis.

Now follows a summary of the results of the series of animal inoculations.

TABLE 1.

Transfer or Subculture	Series	Guinea-Pig Died in	Results
1	4	45 Days	Negri bodies found
2	4	13 "	" " "
3	4	7 "	Meningitis
4	4	8 "	"
5	4	12 "	Negri bodies found
6	4	10 "	" " "

[1] *Jour. Infect. Dis.*, 1906, 3, p. 452.

In the sixth generation occurred a contamination of staphylococcus. An attempt was made to get rid of this by emulsifying the material and passing it through a Berkefeld filter, using the filtrate for further inoculations. This filtrate when incubated showed forms which very closely resembled Negri bodies. A delay in securing sterile media occurred at this time, after which subcultures made from the filtrate were not virulent for animals. Whether the delay or the filtration caused the loss of virulence is not evident.

TABLE 2.

Transfer or Subculture	Series	Guinea-Pig Died in	Results
1	20	10 Days	Negri bodies
2	20	7 "	Meningitis
3	20	12 "	Negri bodies
4	20	5 "	Meningitis
5	20	24 "	Negri bodies

In both these series the controls, which consisted of normal salt solution under oil, inoculated and incubated in the same manner as the cultures, were virulent for guinea-pigs in the second subculture. Animal inoculations with subsequent transfers of the controls gave negative results in every case. This would indicate that enough of the original infective material might be present in the second or third subculture to cause rabies in the animals inoculated. The fact that cultures of the virus in brain medium in the fifth and sixth subculture showed virulence for animals would indicate that the virus had been propagated artificially.

This is not considered as establishing definitely that the artificial cultivation of the virus of rabies has been accomplished, but as encouraging evidence that some modification of the methods described may lead to its successful cultivation.